THE INTERACTION OF PROTEIN AND LIPID IN SONICATED VESICLES OF CYTOCHROME *c* OXIDASE AND PHOSPHATIDYLCHOLINE STUDIED BY ^{1}H NMR SPECTROSCOPY

Karl-Erik FALK and Bo KARLSSON
Institutionen för biokemi och biofysik, Göteborgs universitet och Chalmers tekniska högskola, Fack, S-402 20 Göteborg, Sweden

Received 9 November 1978

1. Introduction

For a full understanding of the properties of membrane-bound proteins, studies on the purified proteins must be performed in a membrane-like environment. In such studies, model membranes of ultrasonicated phospholipids are frequently used. It has been shown that purified cytochrome *c* oxidase can be incorporated into vesicles of egg lecithin, in amounts high enough to allow spectroscopic studies of the enzyme activity in the lipid environment [1]. It is however equally important to investigate the interaction between the protein and lipid since many functions of the protein depend on the lipid environment.

In this study we have used proton NMR spectroscopy to investigate the interaction between cytochrome *c* oxidase and lipid in sonicated small vesicles. The NMR spectrum exhibits 3 well-resolved resonances from the $-N(CH_3)_3^+$, $-(CH_2)_n-$ and $-CH_3$ protons which are sensitive to changes in the dynamics of the vesicle.

We found that these groups of lines showed marked changes in the presence of protein. The integrated intensity of the peaks was reduced down to ~50% in vesicles containing equal amounts (by weight) of oxidase and phospholipid. This is consistent with the idea of a zone of phospholipids close to the protein which is completely immobilized, producing lines too broad to detect. The amount of immobilized lipid is in our investigation calculated to be 0.7 mg lipid/mg oxidase. This figure is higher than what is reported in spin label studies of the motion of the lipids in larger aggregates containing cytochrome *c* oxidase [2–4]. This fact can be ascribed to the different methods used and the different sizes of the vesicles studied.

2. Materials and methods

Cytochrome *c* oxidase was prepared from beef heart mitochondria either by the method in [5] or [6] with subsequent removal of Triton X-100 on a column of Amberlite XAD-2 [7]. L-α-Phosphatidylcholine from egg yolk was purchased from Sigma Chemical Co. Vesicle preparation and incorporation of cytochrome oxidase was made as in [1], except for the buffers, which contained 0.05 M phosphate, 0.1 M KCl in D_2O (pH 7.4) throughout this investigation. Lipid phosphorus was determined by the method in [8] and the protein concentration by its heme content based on cytochrome oxidase mol. wt 200 000. The spectrophotometer used was Beckman model Acta M IV. NMR spectra were obtained by the Fourier transform technique on a Bruker 270 MHz spectrometer at 14°C. A pulse method employing a $90°–\tau–90°$ pulse sequence was used to measure T_1 relaxation times. Integration of the NMR peaks was made using a separate computer (NOVA 3) equipped with a curve follower. All NMR spectra were recorded with the same number of scans and with equal pulse angle and receiver gain to allow for direct magnitude comparison.

3. Results

In the proton NMR spectrum of sonicated egg phosphatidylcholine a triplet structure can be seen in the end methyl group (fig.1A). This structure appears after a sonication time of 15–30 min only in vesicles without any protein present. When cytochrome *c* oxidase was incorporated this structure disappeared already at very small amounts of protein (fig.1B). Apart from this line broadening the integrated intensity of the resonance peaks in the lipid spectrum diminished upon introduction of protein into the bilayer. Judged from diam. measurements of 100 randomly-chosen vesicles [on electron micrographs of the same preparation (cont. 0.1 mg oxidase/mg lipid) used for NMR measurements] vesicle sizes do not change significantly upon introduction of protein. The intensity of the sum of the methyl and the methylene peaks shows a linear decrease up to a protein/lipid weight ratio close to 1 (fig.2). These peaks are overlapping and therefore the sum of their areas was considered more reliable than any of the individual peaks. The curve in fig.2 can be extrapolated to zero NMR intensity at 0.7 mg lipid/mg protein. Reduction of the protein in the presence of CN^- did not give any increase of peak intensity. Measurement of T_1 relaxation times was also performed on the methyl, methylene and choline protons of the lipid spectrum. The largest effect was found on the end methyl groups of the hydrocarbon chains, for which the ratio of the T_1 in presence and in absence of oxidase was found to be 0.8 ± 0.1. The other signals showed ratios which were not significantly different from unity. The T_1 measurements were made on vesicle preparations containing 0.6–0.8 mg protein/mg lipid.

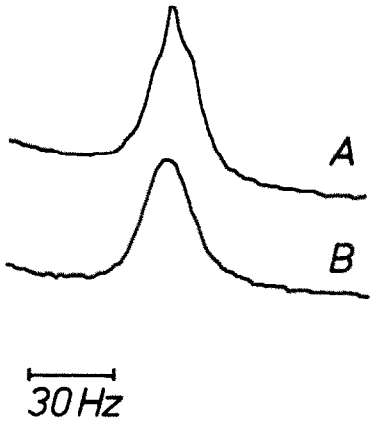

Fig.1. The methyl resonance peak of the 270 MHz ^1H NMR spectrum of phosphatidylcholine in a D_2O buffer medium containing 0.05 M phosphate and 0.1 M potassium chloride at pH 7.4 and 14°C. (A) 13 mg/ml phosphatidylcholine from egg yolk after 30 min sonication. (B) Sample A in the presence of 5.8 μM cytochrome *c* oxidase (mg oxidase/mg lipid = 0.09) after 1 min additional sonication.

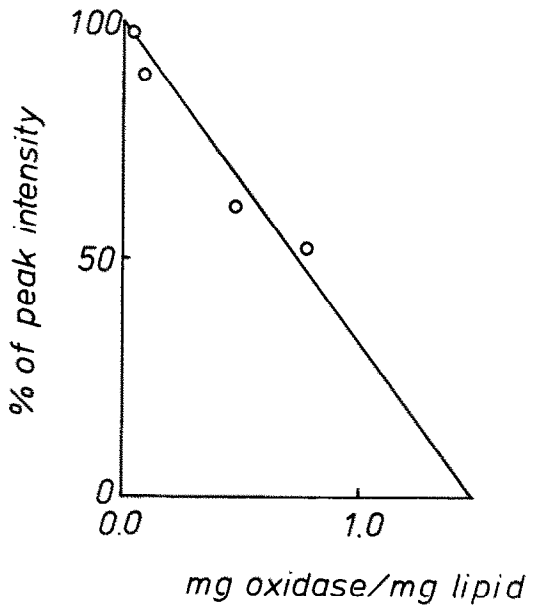

Fig.2. Integrated intensity of methyl + methylene peaks plotted against mg cytochrome *c* oxidase/mg phosphatidylcholine in sonicated vesicles.

4. Discussion

When studying proteins incorporated in artificial membranes one is frequently faced with the problem of measuring the vesicle size. Electron microscopy is of course the obvious choice, but for many reasons an NMR method would be advantageous. The ratio of the inner to outer $-N(CH_3)_3^+$ peaks is in principle useful for this purpose [9], but we have found that the correlation between peak ratios and vesicle size is not simple, especially for the case when protein is incorporated. The resolution of the NMR spectrum is,

however, in general a good indicator of the homogeneity of the vesicle preparation, and we have found that the appearance of the triplet structure in the methyl peak indicates a homogeneous sample of small vesicles. Incorporation of cytochrome *c* oxidase into such phosphatidylcholine vesicles result in a line broadening in the NMR spectrum. At first this is visible at the $-CH_3$ peak, which loses its triplet structure (fig.1). This occurs already at low amounts of cytochrome oxidase (<0.1 mg oxidase/mg lipid). Then the effect on the lipid spectrum is shown as a decrease of intensity, which is proportional to the weight ratio of oxidase/lipid (fig.2). Our interpretation of the decrease of intensity is that part of the phospholipids, in a layer close to the oxidase molecules, are immobilized, producing lines too broad to detect in the NMR spectrum. The effects can not be explained by paramagnetic broadening from the oxidase, since vesicles containing diamagnetic protein, i.e., reduced in the presence of CN^- [10], did not show a significantly different spectrum than vesicles containing the same amount of oxidized protein. The calculated amount of immobilized lipid is higher than that found [2,3] using EPR spectroscopy with a spin label in a natural mitochondrial lipid mixture. The difference can be ascribed to the different composition of lipids and the different methods used. The motion dependence for the spin label EPR lines and the NMR lines is difficult to quantitate for the rather complicated vesicle system but that the above mentioned results are reasonable can be understood from the observation that large vesicles ($\simeq$100 nm) give spin label EPR lines typical of a fast motion situation, whereas an NMR spectrum exhibits a broad feature [11].

At an even higher content of protein (mg protein/mg lipid>1) the intensity of the signals did not continue to decrease lineraly. This could reflect the difficulty of incorporating cytochrome oxidase in amounts close to the total protein content in the mitochondrial membrane. This suggestion is supported by electron micrographs of such vesicle preparations, which showed oxidase molecules that were not incorporated, in the buffer solution outside the vesicles.

The phospholipids which were visible in the NMR spectrum were also perturbed by the presence of the protein. The T_1 relaxation time for the $-CH_3$ protons at the end of the hydrocarbon chains was considerably shortened, indicating that the lateral diffusion or the local segmental motion was hindered. This result shows that cytochrome oxidase has the greatest effect on resonances originating from protons in the hydrophobic interior of the bilayer. This is clearly different from the effects of cytochrome *c* which gave the largest changes in T_1 values for protons near the membrane surface [12].

The present investigation on sonicated lipid vesicles using proton NMR spectroscopy gives support to the interpretation made earlier [2–4] that the oxidase molecule is surrounded by a zone of immobilized lipid, which by our method is estimated to be about 3 molecular layers.

Acknowledgements

We wish to thank Professor B. G. Malmström and Professor T. Vänngård for valuable advice and stimulating discussions during this work. We thank Dr G. Berg in the Department of Anatomy, University of Göteborg for running the electron microscopy experiments, and Mr Per Lincoln for determinations of lipid phosphorus. This work was supported by grants from the Swedish Natural Science Research Council (K 2131-030 and F 2214-021), Axel and Margaret Ax:son Johnsson Foundation, Torsten and Ragnar Söderberg Foundation, Knut and Alice Wallenberg Foundation and the Tercentenary Fund of the Bank of Sweden.

References

[1] Karlsson, B., Lanne, B., Malmström, B. G., Berg, G. and Ekholm, R. (1977) FEBS Lett. 84, 291–295.
[2] Jost, P. C., Griffith, O. H., Capaldi, R. A. and Vanderkooi, G. (1973) Proc. Natl. Acad. Sci. USA 70, 480–484.
[3] Jost, P. C., Nadakavukaren, K. K. and Griffith, O. H. (1977) Biochemistry 16, 3110–3114.
[4] Marsh, D., Watts, A., Maschke, W. and Knowles, P. F. (1978) Biochem. Biophys. Res. Commun. 81, 397–402.
[5] Van Buuren, K. J. H. (1972) Binding of Cyanid to Cytochrome aa_3, Ph.D. Thesis, University of Amsterdam, Gerja, Waarland.

[6] Rosén, S. (1978) Biochim. Biophys. Acta 523, 314–320.
[7] Holloway, P. W. (1973) Anal. Biochem. 53, 304–308.
[8] Chen, P. S., Toribara, T. Y. and Warner, H. (1957) Anal. Chem. 28, 1756–1758.
[9] Kostelnik, R. J. and Castellano, S. M. (1973) J. Magn. Res. 9, 291–295.
[10] Falk, K.-E., Vänngård, T. and Angström, J. (1977) FEBS Lett. 75, 23–27.
[11] Sheetz, P. M. and Chan, S. I. (1972) Biochemistry 11, 4573–4581.
[12] Brown, L. R. and Wütrich, K. (1977) Biochim. Biophys. Acta 468, 389–410.